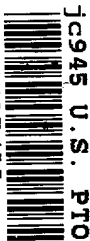


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PTO/SB/05 (1/98)

Approved for use through 09/30/2000. OMB 0651-0032

Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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<b>UTILITY PATENT APPLICATION TRANSMITTAL</b> (Only for new nonprovisional applications under 37 CFR 1.53(b))	Attorney Docket No.	4555US
	First Inventor or Application Identifier	Stefan M.C. Pype et al.
	Title	See 1 in Addendum
	Express Mail Label No.	EL700257102US

<b>APPLICATION ELEMENTS</b> See MPEP chapter 600 concerning utility patent application contents.	<b>ADDRESS TO:</b> Assistant Commissioner for Patents Box Patent Application Washington, DC 20231
1. <input checked="" type="checkbox"/> * Fee Transmittal Form (e.g., PTO/SB/17) (Submit an original, and a duplicate for fee processing)	6. <input type="checkbox"/> Microfiche Computer Program (Appendix)
2. <input checked="" type="checkbox"/> Specification [Total Pages 42] (preferred arrangement set forth below) <ul style="list-style-type: none"><li>- Descriptive title of the invention</li><li>- Cross References to Related Applications</li><li>- Statement Regarding Fed sponsored R &amp; D</li><li>- Reference to Microfiche Appendix</li><li>- Background of the invention</li><li>- Brief Summary of the invention</li><li>- Brief Description of the Drawings (if filed)</li><li>- Detailed Description</li><li>- Claim(s)</li><li>- Abstract of the Disclosure</li></ul>	7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary) <ul style="list-style-type: none"><li>a. <input checked="" type="checkbox"/> Computer Readable Copy</li><li>b. <input checked="" type="checkbox"/> Paper Copy (identical to computer copy)</li><li>c. <input checked="" type="checkbox"/> Statement verifying identity of above copies</li></ul>
3. <input checked="" type="checkbox"/> Drawing(s) (35 U.S.C. 113) [Total Sheets 2]	<b>ACCOMPANYING APPLICATION PARTS</b> <ul style="list-style-type: none"><li>8. <input type="checkbox"/> Assignment Papers (cover sheet &amp; document(s))</li><li>9. <input type="checkbox"/> 37 C.F.R. § 3.73(b) Statement (when there is an assignee) <input type="checkbox"/> Power of Attorney</li><li>10. <input type="checkbox"/> English Translation Document (if applicable)</li><li>11. <input checked="" type="checkbox"/> Information Disclosure Statement (IDS)/PTO-1449 <input checked="" type="checkbox"/> Copies of IDS Citations</li><li>12. <input checked="" type="checkbox"/> Preliminary Amendment</li><li>13. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) (Should be specifically itemized)<ul style="list-style-type: none"><li>* Small Entity Statement(s) <input type="checkbox"/> Statement filed in prior application, Status still proper and desired (PTO/SB/09-12)</li></ul></li><li>14. <input type="checkbox"/> Certified Copy of Priority Document(s) (if foreign priority is claimed)</li><li>15. <input type="checkbox"/> Other: .....</li><li>16. <input type="checkbox"/> Other: .....</li></ul> <p>* A new statement is required to be entitled to pay small entity fees, except where one has been filed in a prior application and is being relied upon.</p>
4. Oath or Declaration [Total Pages ] <ul style="list-style-type: none"><li>a. <input type="checkbox"/> Newly executed (original or copy)</li><li>b. <input type="checkbox"/> Copy from a prior application (37 C.F.R. § 1.63(d)) (for continuation/divisional with Box 17 completed) [Note Box 5 below]<ul style="list-style-type: none"><li>i. <input type="checkbox"/> <b>DELETION OF INVENTOR(S)</b> Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).</li></ul></li></ul>	
5. <input checked="" type="checkbox"/> Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	
17. If a <b>CONTINUING APPLICATION</b> , check appropriate box, and supply the requisite information below and in a preliminary amendment: <input checked="" type="checkbox"/> Continuation <input type="checkbox"/> Divisional <input type="checkbox"/> Continuation-in-part (CIP) of prior application No: PCT/JP99/03025 Prior application information: Examiner _____ Group / Art Unit: _____	

<b>18. CORRESPONDENCE ADDRESS</b>			
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Name (Print/Type)	Allen C. Turner	Registration No. (Attorney/Agent)	33,041
Signature		Date	10/27/00

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# FEE TRANSMITTAL

## for FY 2000

Patent fees are subject to annual revision.

Small Entity payments must be supported by a small entity statement, otherwise large entity fees must be paid. See Forms PTO/SB/09-12. See 37 C.F.R. §§ 1.27 and 1.28.

TOTAL AMOUNT OF PAYMENT (\$ 355.00)

## Complete if Known

Application Number	
Filing Date	October 27, 2000
First Named Inventor	Stefan M.C. Pye et al.
Examiner Name	
Group / Art Unit	
Attorney Docket No.	4555US

## METHOD OF PAYMENT (check one)

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

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Deposit Account Name Trask Britt

☒ Charge Any Additional Fee Required Under 37 CFR §§ 1.16 and 1.17

2. ☒ Payment Enclosed:

☒ Check ☐ Money Order ☐ Other

## FEE CALCULATION

## 1. BASIC FILING FEE

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
101	690	201	355	Utility filing fee	355
106	310	206	155	Design filing fee	0
107	480	207	240	Plant filing fee	0
108	690	208	345	Reissue filing fee	0
114	150	214	75	Provisional filing fee	0

SUBTOTAL (1) (\$ 355.00)

## 2. EXTRA CLAIM FEES

Total Claims	Extra Claims	Fee from below	Fee Paid
16	-20** = 0	9	0
3	-3** = 0	39	0
Multiple Dependent		0	0

\*\*or number previously paid, if greater; For Reissues, see below

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
103	18	203	9	Claims in excess of 20	
102	78	202	39	Independent claims in excess of 3	
104	260	204	130	Multiple dependent claim, if not paid	
109	78	209	39	** Reissue independent claims over original patent	
110	18	210	9	** Reissue claims in excess of 20 and over original patent	

SUBTOTAL (2) (\$ 0.00)

## FEE CALCULATION (continued)

## 3. ADDITIONAL FEES

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	380	216	190	Extension for reply within second month	
117	870	217	435	Extension for reply within third month	
118	1,360	218	680	Extension for reply within fourth month	
128	1,850	228	925	Extension for reply within fifth month	
119	300	219	150	Notice of Appeal	
120	300	220	150	Filing a brief in support of an appeal	
121	260	221	130	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,210	241	605	Petition to revive - unintentional	
142	1,210	242	605	Utility issue fee (or reissue)	
143	430	243	215	Design issue fee	
144	580	244	290	Plant issue fee	
122	130	222	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	690	246	345	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	690	249	345	For each additional invention to be examined (37 CFR § 1.129(b))	

Other fee (specify) \_\_\_\_\_

Other fee (specify) \_\_\_\_\_

\* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) (\$ )

## SUBMITTED BY

Name (Print/Type)	Allen C. Turner	Registration No. (Attorney/Agent)	33,041	Telephone	(801) 532-1922
Signature		Date	10/27/2000		

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00697563 40600

**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**In re Application of:**

Pype et al.

**Serial No.:** To be assigned

**Filed:** October 27, 2000

**For:** CD40-INTERACTING AND  
TRAF-INTERACTING PROTEINS

**Examiner:** To be assigned

**Group Art Unit:** To be assigned

**Attorney Docket No.:** 4555US

**NOTICE OF EXPRESS MAILING**

Express Mail Mailing  
Label Number: EL700257102US

Date of Deposit with USPS: October 27, 2000

Person making Deposit: Jared Turner

**PRELIMINARY AMENDMENT**

Box Patent Application  
Commissioner for Patents  
Washington, D.C. 20231

Sir:

Before calculating the filing fee, please amend the referenced application as follows:

**IN THE CLAIMS:**

1. (Amended) An isolated [functional] protein capable of interacting with [the cytoplasmic domain of CD40 and/or other] receptors of the TNF superfamily [such as] including the cytoplasmic domain of CD40, CD30 and TNF receptor II [wherein said protein has no homology to TRAF-proteins] said isolated protein comprising an amino acid sequence having 70-100% homology to the amino acid sequence depicted in SEQ ID NO. 2 or a fragment thereof.

Please cancel claim 2.

3. (Amended) [An]The isolated [functional] protein [according to]of claim 1 further comprising [an] the amino acid sequence [with 70-100% homology to the amino acid sequence] depicted in SEQ ID NO. 4.

Please cancel claim 4.

5. (Amended) [An] isolated [functional] protein [according to] claim 1 [comprising an amino acid sequence with 70-100% homology to] wherein said isolated protein is a fragment, said fragment comprising the amino acids 54-362 of SEQ ID NO. 2.

6. (Amended) [An]The isolated [functional] protein [according to] claim [5] 1 [comprising an amino acid sequence with 70-100% homology to] wherein said isolated protein is a fragment comprising the amino acids 274-362 of SEQ ID NO. 2.

Please cancel claim 7

8. (Amended) A nucleic acid sequence encoding a protein according to [any of the claims 1-7] claim 1.

9. (Amended) [A] The nucleic acid sequence [according to]of claim 8 with 70-100% homology to the DNA sequence depicted in SEQ ID NO. 1.

10. (Amended) [A] The nucleic acid sequence [according to] of claim [8] 9 with 70-100% homology to the DNA sequence depicted in SEQ ID NO. 3.

Please cancel claim 11.

12. (Amended) [Use] A method of modulating CD40 signaling pathways of a subject, the method comprising:

providing to the subject a pharmaceutically acceptable amount of an isolated [functional] protein [and/]or a [functional] fragment thereof [according to any of the claims 1-7 as a medicament] comprising an amino acid sequence with 70-100% homology to the amino acid sequence depicted in SEQ ID NO. 2 or a fragment thereof.

13. [Use of an isolated functional protein] The method according to [any of the claims 1-7 and/or a functional fragment thereof to treat] claim 12 wherein the modulation of CD40 signaling pathways comprises the treatment of a disease selected from the group of diseases consisting of TRAF-related, CD40-related, NF-KB related and[/or] Jun (kinase)-related diseases.

14. (Amended) The [use] method according to claim 13[ in which], wherein the disease is selected from the group consisting of atherosclerosis, [arthritis] arthritis, multiple sclerosis, systemic lupus [erythematosus]erythematosus and[/or] graft rejection.

15. (Amended) The [use of an isolated functional protein] method according to [any of the claims 1-7 and/or a functional fragment thereof to sensitize] claim 12 wherein the modulation of CD40 signaling pathways sensitizes tumor cells to anti-tumor treatments.

16. (Amended) [The use of an]A method of screening for compounds that interfere with the interaction of the isolated [functional] protein [according to of the claims 1-7]of claim 1 and/or a functional fragment thereof [to screen for compounds that interfere with the interaction of said protein(s)] with other protein components of the TRAF, CD40 or NF-KB related pathway, said method comprising:

interacting said isolated protein and/or functional fragment thereof with said other protein components, and  
detecting the compounds' affect on said interaction.

17. (Amended) A method for screening compounds, said method comprising the use of a protein according to claim [16] 1 to screen for compounds that modulate the activity of CD40 receptor associated proteins.

18. (Amended) A compound [isolated with the method] produced by the method according to claim [17]16.

19. (Amended) A pharmaceutical composition comprising one or more isolated functional proteins [according to any of the claims 1-7]claim 1 and/or functional fragments thereof and a pharmaceutical acceptable carrier material..

Please cancel claim 21.

Please add the following new claims:

22. A method of treating CD40 and NF-KB related diseases selected from the group of diseases consisting of atherosclerosis, arthritis, multiple sclerosis, systemic lupus erythematosus and graft rejection, the method comprising:

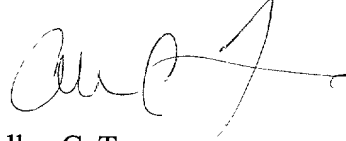
providing to a subject an effective amount of an isolated protein comprising an amino acid sequence with 70-100% homology to the amino acids 274-362 of SEQ ID NO. 2.

#### **Remarks**

The application is to be amended as previously set forth. All amendments, including claim cancellations, are made without prejudice or disclaimer. The amendments are made to bring the application closer to United States practice, such as, by example, removing multiple claim dependencies. It is respectfully submitted that no new matter has been added by the amendment.

If questions exist after consideration of the foregoing, the Office is kindly requested to contact the applicants' representative at the address or telephone number below.

Respectfully submitted,



Allen C. Turner  
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Attorney for Applicants  
TRASK BRITT  
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Salt Lake City, Utah 84110-2550  
Telephone: (801) 532-1922

Date: October 27, 2000

## Addendum

### 1. CD40-INTERACTING AND TRAF-INTERACTING PROTEINS

09/23/2014 10:43:25 AM



PATENT

Attorney Docket 4555US

CERTIFICATE OF MAILING

Express Mail Label Number: EL700257102US

Date of Deposit: October 27, 2000

Person making Deposit: Jared Turner

PATENT APPLICATION

for

CD40-INTERACTING AND TRAF-INTERACTING PROTEINS

Inventors:

Stefan M. C. Pype  
Jacques E. F. Remacle  
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## CD40-INTERACTING AND TRAF-INTERACTING PROTEINS

Cross-reference to related application: This application is a continuation of co-pending International Application No. PCT/EP99/03025, filed on April 28, 1999, designating the United States of America, the contents of which are incorporated by this reference, which  
5 itself claims priority from European Patent Application EP 98201392.2, filed on April 29, 1998.

Technical Field: The invention relates to CD40 binding proteins, which can be used as modulators of the CD40 signaling pathway and/or the CD40-induced nuclear factor kappa B (NF- $\kappa$ B) activating pathway and thus useful in the treatment of CD40 related diseases  
10 (*e.g.*, inflammatory diseases) and/or NF- $\kappa$ B related diseases and/or in the improvement of anti-tumor treatments. The current invention also relates to nucleic acid sequences coding for the CD40 interacting proteins (also called "TTRAP" for CD40 receptor associated protein). The invention further relates to the use of the polypeptides derived from these CD40 interacting proteins in the treatment of CD40 and/or NF- $\kappa$ B related diseases and/or  
15 cancer. Furthermore, the invention concerns pharmaceutical preparations comprising the CD40 interacting proteins or polypeptides derived from these proteins.

Background: CD40 is a receptor of the TNF- receptor superfamily (Banchereau *et al.*, 1994), which is expressed at the surface of B-cells, antigen presenting cells (APC), and several non-hematopoietic cells such as endothelial cells (Hollenbaugh *et al.*, 1995),  
20 epithelial cells (Galy & Spits, 1992), fibroblasts (Fries *et al.*, 1995) and keratinocytes (Gaspari *et al.*, 1996). The ligand for CD40 (CD40L) occurs mainly on activated T-cells. Up to now the role of CD40 was mainly studied in the context of the T-cell APC / B-cell interaction (for a review, see Noelle, 1996). Amongst others, the CD40-CD40L interaction seems to be important for the T-cell mediated immunity and for primary and secondary  
25 humoral immune response. These findings were confirmed by experiments in mouse models showing that treatment with anti-CD40L antibodies resulted in blocking of the development of mouse equivalents of human autoimmune diseases such as arthritis (Durie *et al.* 1993), oophoritis (Griggs *et al.*, 1996) and multiple sclerosis (Gerritse *et al.*, 1996).

Activation and transduction through the CD40 pathway within this biological system is mainly responsible for B cell activation and the humoral immune response accordingly.

Apart from NF- $\kappa$ B, factors that can be activated by stimulation of CD40 are NF-AT (Francis *et al.*, 1995) c-Jun, ATF-2 and IRF-1 (Karmann *et al.*, 1996). All these factors play an important role in inflammation.

The CD40L induced signal transduction is, like TNF, mediated by the binding of TNF-Receptor Associated Factors (TRAF's) to the cytoplasmic domain of the receptor. Chaudhuri *et al.* (1997) demonstrated that, at least in human B cell lines, CD40 and TRAF2 are constitutively associated with each other, and that this association is inhibited by CD40 mediated signals. Apart from the binding with TRAF2, the cytoplasmic domain of CD40, which consists of 62 amino acids at positions 196-257 (mature human CD40 - numbering according to Kashiwada *et al.*, 1998), is known to associate with TRAF3, TRAF5, TRAF6 and Janus kinase 3. TRAF6 binds to the amino-terminal cytoplasmic tail of CD40 at positions 210-225, although it can not be excluded that full association of TRAF6 with CD40 may also require the carboxy-terminal part at positions 226-249 (Ishida *et al.*, 1996). TRAF2, TRAF3 and TRAF5 bind to the carboxy-terminal CD40 cytoplasmic domain at positions 226-249 (Ishida *et al.*, 1996).

Stimulation of CD40 results in activation of protein kinases, the mitogen-activated protein kinase and Janus kinase 3 / signal transducer and activator of Transcription 3. Moreover, stimulation of CD40 mediates critical biological effects in B cell growth, survival and differentiation.

It is known that TRAF2 and TRAF5 play a role in NF- $\kappa$ B activation in signaling through CD40, as well as TNF-RI, TNF-RII, CD30 and lymphotoxin b receptor. TRAF6 participates in NF- $\kappa$ B activation signaled by CD40 and IL-1 receptor. In addition to these data, International Patent Applications WO 96/16665 and WO 96/28568 disclose a TRAF like protein that binds to the cytoplasmic domain of CD40.

### **Description of the Invention**

We show herein that at least two other proteins exist which unexpectedly interact with the cytoplasmic domain of CD40. Even more surprisingly, neither of these proteins

shows significant homology with any known CD40 interacting proteins. Further, no significant homology exists between the two proteins themselves. These proteins should therefore be considered as two new classes of CD40 interacting proteins.

The present invention thus concerns an isolated functional protein capable of interacting with the cytoplasmic domain of CD40 and/or other receptors of the TNF receptor superfamily such as CD30 or TNF receptor II, wherein the protein has no homology to TRAF-proteins.

The invention also includes an isolated functional protein either comprising an amino acid sequence with 70-100% homology to the amino acid sequence depicted in SEQ ID NO. 2 or either comprising an amino acid sequence with 70-100% homology to the amino acid sequence depicted in SEQ ID NO. 4 or in the alternative comprising an amino acid sequence with 70-100% homology to the amino acid sequence depicted in SEQ ID NO. 6.

More specifically, the functional protein comprises an amino acid sequence with 70-100% homology to the amino acids 54-362 of SEQ ID NO. 2, even more specifically the functional protein comprises an amino acid sequence with 70-100% homology to the amino acids 274-362 of SEQ ID NO. 2 or in the alternative and/or comprising an amino acid sequence with 70-100% homology to the amino acids 2-245 of SEQ ID NO.6.

Furthermore, the invention also includes those proteins or peptides having 70-100% homology to, at least, any of the three peptides as depicted in SEQ.ID.NO.2 located between the residues 115-121, 145-153 and 347-352 respectively. The amino acid sequence of residue numbering 115-121 is SLITWNI; the amino acid sequence of residue numbering 145-153 is PDVIFLQEV and the amino acid sequence of residue numbering 347-352 is FPSDHW.

“Homology”, in this context, means identical or similar to the referenced sequence while obvious replacements/modifications of any of the amino acids provided, are included as well. A homology search in this respect can be performed with the BLAST-P (Basic Local Alignment Search Tool), a program well known to those of skill in the art. For the corresponding nucleic acid sequence, homology is referred to the BLASTX and BLASTN programs known in the art.

The invention thus relates to a DNA sequence encoding a CD40 receptor associated protein or encoding an immunologically active and/or functional fragment of such a protein, selected from the group consisting of:

- (a) DNA sequences comprising a nucleotide sequence encoding a protein comprising the amino acid sequence as given in SEQ ID NO: 2;
- (b) DNA sequences comprising a nucleotide sequence as given in SEQ ID NO: 1;
- (c) DNA sequences hybridizing with the complementary strand of a DNA sequence as defined in (a) or (b) and encoding an amino acid sequence which is at least 70% identical to the amino acid sequence encoded by the DNA sequence of (a) or (b);
- (d) DNA sequences, the nucleotide sequence of which is degenerated as a result of the genetic code to a nucleotide sequence of a DNA sequence as defined in any one of (a) to (c); and
- (e) DNA sequences encoding a fragment of a protein encoded by a DNA sequence of any one of (a) to (d).

One embodiment of the invention is a protein with SEQ ID NO.2. Another embodiment of the invention is a protein with SEQ ID NO.4. A further embodiment of the invention concerns a protein with SEQ ID NO.6.

A further aspect of the invention is the use of the aforementioned proteins, or biologically active fragments thereof, to modulate and/or inhibit members of the TNF receptor superfamily such as CD40, CD30 or TNF-receptor II in their signaling activity and/or CD40-induced NF-kB activation and/or JUN-kinase activity.

The isolated functional protein according to the invention and/or a functional fragment thereof can be used to treat TRAF-related, CD40-related, NF-kB related and/or Jun (kinase) related diseases. Such diseases include atherosclerosis, arthritis, multiple sclerosis, systemic lupus erythematosus ("SLE") and/or graft rejection.

In addition, the isolated functional protein according to the invention and/or a functional fragment thereof can be used to sensitize tumor cells to anti-tumor treatments.

Another aspect of the invention is the use of aforementioned proteins or biologically active fragments thereof to screen for compounds that interfere in the interactions of the proteins or fragments with other protein components of the CD40, TRAF or NF-kB related signaling pathway.

The invention also relates to a method for identifying and obtaining an activator or inhibitor of CD40 receptor associated proteins comprising the steps of:

- (a) combining a compound to be screened with a reaction mixture containing the protein of the invention and a readout system capable of interacting with the protein under suitable conditions;
- (b) maintaining the reaction mixture in the presence of the compound or a sample comprising a plurality of compounds under conditions which permit interaction of the protein with the read out system;
- (c) identifying or verifying a sample and compound, respectively, which leads to suppression or activation of the read out system.

As used herein, the term "read out system" means a DNA sequence which upon transcription and/or expression in a cell, tissue or organism provides for a scorable and/or selectable phenotype. Such read out systems are well known to those skilled in the art and comprise, for example, recombinant DNA molecules and marker genes as previously described herein.

The term "plurality of compounds" in a method of the invention is to be understood as a plurality of substances which may or may not be identical.

The compound or plurality of compounds may be comprised in, for example, samples, *e.g.*, cell extracts from animals or microorganisms. Furthermore, the compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating CD-40 receptor associated interacting proteins. The reaction mixture may be a cell free extract or may comprise a cell or tissue culture. Suitable set ups for the method of the invention are known to the person skilled in the art and are, for example, generally described in Alberts et al., Molecular Biology of the Cell, (3<sup>rd</sup> ed. 1994). The plurality of compounds may be, *e.g.*, added to the reaction mixture, culture medium or injected into the cell.

If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating CD40 receptor associated proteins, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably, the sample comprises substances of similar chemical and/or physical properties, and most preferably the substances are identical. The compounds which can be tested and identified according to a method of the invention may be expression libraries, *e.g.*, cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198 and references cited *supra*).

Another aspect of the invention involves DNA molecules, also called nucleic acid sequences, encoding for the aforementioned proteins, preferably a nucleic acid sequence with 70-100% homology to the DNA sequence depicted in SEQ ID NO. 1 and/or a nucleic acid sequence with 70-100% homology to the DNA sequence depicted in SEQ ID NO.3 or in the alternative a nucleic acid sequence with 70-100% homology to the DNA sequence depicted in SEQ ID NO. 5.

“Homology”, in this context, means that the respective nucleic acid molecules or encoded proteins are functionally and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of the nucleic acid molecules are, for example, variations of the nucleic acid molecules which represent modifications having the same biological function, in particular encoding proteins with the same or substantially the same biological function. They may be naturally occurring variations, such as sequences from other varieties or species, or mutations. These mutations may occur naturally or may be obtained by mutagenesis techniques. The allelic variations

may be naturally occurring allelic variants as well as synthetically produced or genetically engineered variants.

The proteins encoded by the various derivatives and variants of the above-described nucleic acid molecules have similar common characteristics, such as biological activity, molecular weight, immunological reactivity, conformation, etc., as well as physical properties, such as electrophoretic mobility, chromatographic behavior, sedimentation coefficients, pH optimum, temperature optimum, stability, solubility, spectroscopic properties, etc.

A further embodiment of the invention concerns a method for identifying and obtaining CD40 receptor associated proteins comprising the LexA two-hybrid system wherein LexA DNA-binding domain as a bait and a HeLa cell fusion library in plasmid pJG45 as prey is used. A DNA sequence encoding a CD40 receptor associated protein obtainable by the method belong to the invention as well.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain a nucleic acid molecule according to the invention. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y.

Alternatively, the nucleic acid molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

In a preferred embodiment, the nucleic acid molecule present in the vector is operably linked to a control sequence or control sequences that allow for the expression of the nucleic acid molecule in prokaryotic and/or eukaryotic cells.

The term "control sequence" refers to regulatory DNA sequences which are necessary to affect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In procaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eucaryotes generally, control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended



to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. If the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is used.

Thus, the vector of the invention is preferably an expression vector. An "expression vector" is a construct that can be used to transform a selected host cell and provides for expression of a coding sequence in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art. The present invention furthermore relates to host cells comprising a vector as described above or a nucleic acid molecule according to the invention wherein the nucleic acid molecule is foreign to the host cell.

By "foreign" it is meant that the nucleic acid molecule is either heterologous with respect to the host cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of the nucleic acid molecule. This means that, if the nucleic acid molecule is homologous with respect to the host cell, it is not located in its natural location in the genome of the host cell, in particular it is surrounded by different genes. In this case the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The vector or nucleic acid molecule according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extra-chromasomally. In this respect, it is also to be understood that the nucleic acid molecule of the invention can be used to restore or create a mutant gene via homologous recombination (Paszkowski (ed.),

Homologous Recombination and Gene Silencing in Plants. Kluwer Academic Publishers (1994)).

The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus *Saccharomyces*, in particular those of the species *S. cerevisiae*.

The invention also includes a method for preparing CD40 receptor associated proteins which method comprises the cultivation of host cells according to the invention which, due to the presence of a vector or a nucleic acid molecule according to the invention, are able to express such a protein, under conditions which allow expression of the protein and recovering of the so-produced protein from the culture.

The present invention furthermore relates to proteins encoded by the nucleic acid molecules according to the invention or produced or obtained by the herein described methods, and to functional and/or immunologically active fragments of such CD40 receptor associated proteins. The proteins and polypeptides of the present invention are not necessarily translated from a designated nucleic acid sequence; the polypeptides may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system, or isolation from a suitable viral system. The polypeptides may include one or more analogs of amino acids, phosphorylated amino acids or unnatural amino acids. Methods of inserting analogs of amino acids into a sequence are known in the art. The polypeptides may also include one or more labels, which are known to those skilled in the art. In this context, it is also understood that the proteins according to the invention may be further modified by conventional methods known in the art. By providing the proteins according to the present invention it is also possible to determine fragments which retain biological activity, namely the mature, processed form. This allows the construction of chimeric proteins and peptides comprising an amino sequence derived from the protein of the invention, which is crucial for its binding activity. The other functional amino acid sequences may be either physically linked by, *e.g.*, chemical means to the proteins of the invention or may be fused by recombinant DNA techniques well known in the art.

Furthermore, the present invention relates to antibodies specifically recognizing a CD40 receptor associated protein according to the invention or parts, *i.e.*, specific fragments

or epitopes, of such a protein. The antibodies of the invention can be used to identify and isolate other CD40 receptor associated proteins and genes in any organism. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, *e.g.*, in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. These antibodies can be used, for example, for the immunoprecipitation and immunolocalization of proteins according to the invention as well as for the monitoring of the synthesis of such proteins, for example, in recombinant organisms, and for the identification of compounds interacting with the protein according to the invention. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies selections, yielding a high increment of affinity from a single library of phage antibodies which bind to an epitope of the protein of the invention (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmberg, J. Immunol. Methods 183 (1995), 7-13). In many cases, the binding phenomena of antibodies to antigens is equivalent to other ligand/anti-ligand binding.

The invention also relates to a diagnostic composition comprising at least one of the aforementioned nucleic acid molecules, vectors, proteins, antibodies or compounds and optionally suitable means for detection.

The diagnostic compositions may be used for methods for detecting expression of related CD40 receptor associated proteins by detecting the presence of the corresponding mRNA which comprises isolation of mRNA from a cell and contacting the mRNA so obtained with a probe comprising a nucleic acid probe as described above under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the protein in the cell. Further methods of detecting the presence of a protein according to the present invention comprises immuno-techniques well known in the art, for example enzyme linked immunosorbent assay.

The invention also relates to a pharmaceutical composition comprising one or more of the above mentioned proteins or fragments in a biologically active amount for the treatment of CD40, TRAF and/or NF-kB related diseases such as atherosclerosis, arthritis, multiple sclerosis, systemic lupus erythematosus, graft rejection and the like.

5 In another aspect, the present invention relates to a pharmaceutical composition comprising one or more compounds obtainable by the above mentioned screening method for the treatment of CD40, TRAF and/or NF-kB related diseases such as atherosclerosis, arthritis, multiple sclerosis, systemic lupus erythematosus, graft rejection and the like.

### Brief Description of The Figures

10 **FIG. 1:** schematic representation of TTRAP (=CD40 receptor associated protein) and the deletion mutants of TTRAP used in two-hybrid assays. The deletion mutants consist of the following amino acids of the original TTRAP sequence: 54 to 362 (4F2), 54 to 273 (4F2d3), 54 to 236 (4F2d2) and 54 to 140 (4F2d1). ( CaM= calcium calmodulin binding region).

15 **FIG. 2:** Northern blot analysis of (a) human tissue, using a human TTRAP probe; (b) adult mouse tissue, using a mouse TTRAP probe; (c) embryonic mouse tissue, using a mouse TTRAP probe. The hybridization of GAPDH is used as a control.

### Detailed Description of the Invention

#### DEFINITIONS

20 The following definitions are provided in order to further illustrate and define the meaning and scope of the various terms used in the current description.

As used herein, "homology to TRAF-proteins" means that the typical structural features found in the current TRAF proteins (TRAF1 - TRAF6) are present. These features comprise a RING finger motif at the amino terminus followed by five or more zinc fingers  
25 and a so-called TRAF domain known to a person skilled in the art.

As used herein, "CD40", it is not to limit the scope to CD40 only, but also includes other receptors of the TNF receptor superfamily such as CD30 or TNF receptor II, unless indicated to the contrary.

The term "treatment" or "treating" or "treat" means any treatment of a disease in a mammal, including : (1) preventing the disease causing the clinical symptoms of the disease not to develop; (2) inhibiting the disease arresting the development of the clinical symptoms; and/or (3) relieving the disease causing the regression of clinical symptoms.

The term "effective amount" means a dosage sufficient to provide treatment for the disease state being treated. This will vary depending on the patient, the disease and the treatment being effected.

"Capable of interacting" means that a protein can form a complex with another protein, as can be measured using a yeast two-hybrid system, or with co-immunoprecipitation, or with equivalent systems known to people skilled in the art.

"Functional" protein or fragment means a protein or fragment that is capable to interact with the cytoplasmic part of CD40, or with another protein of the CD40 and/or NF-kB related pathway.

The "cytoplasmic part of CD40" means a part comprising the 62 carboxy terminal amino acids of human CD40 (amino acid 216-277; Stamenkovic *et al.* 1989), or the homologous mouse sequence, or another homologous sequence with a similar biological activity.

The term "expression" means the production of a protein or nucleotide sequence in the cell. However, the term also includes expression of the protein in a cell-free system. It includes transcription into an RNA product, post-transcriptional modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications. Depending on the specific constructs and conditions used, the protein may be recovered from the cells, from the culture medium or from both. For the person skilled in the art it is well known that it is not only possible to express a native protein but also to express the protein as fusion polypeptides or to add signal sequences directing the protein to specific compartments of the host cell, *e.g.*, ensuring secretion of the peptide into the culture medium, etc. Furthermore, such a protein and

fragments thereof can be chemically synthesized and/or modified according to standard methods.

The terms "protein" and "polypeptide", as used herein, are interchangeable. "Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus, peptides and oligopeptides are included within the definition of polypeptide. This term also refers to or includes post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog. Preferably, the DNA sequence of the invention comprises a coding sequence encoding the above defined CD40 receptor associated protein.

A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

The term "functional fragment of a sequence" or "functional part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity of the original sequence referred to, while the maximum size is not critical. In some

applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid sequence will range from about 5 to about 60 amino acids in length. More typically, however, the sequence will be a maximum of about 50 amino acids in length, preferably a maximum of about 30 amino acids. It is usually desirable to select sequences of at least about 10, 12 or 15 amino acids, up to a maximum of about 20 or 25 amino acids.

Furthermore, folding simulations and computer redesign of structural motifs of the protein of the invention can be performed using appropriate computer programs (Olszewski, *Proteins* 25 (1996), 286-299; Hoffman, *Comput. Appl. Biosci.* 11 (1995), 675-679).

Computer modeling of protein folding can be used for the conformational and energetic analysis of detailed peptide and protein models (Monge, *J. Mol. Biol.* 247 (1995), 995-1012; Renouf, *Adv. Exp. Med. Biol.* 376 (1995), 37-45). In particular, the appropriate programs can be used for the identification of interactive sites of the inventive protein, its receptor, its ligand or other interacting proteins by computer assistant searches for complementary peptide sequences (Fassina, *Immunomethods* 5 (1994), 114-120. Further appropriate computer systems for the design of protein and peptides are described in the prior art, for example in Berry, *Biochem. Soc. Trans.* 22 (1994), 1033-1036; Wodak, *Ann. N. Y. Acad. Sci.* 501 (1987), 1-13; Pabo, *Biochemistry* 25 (1986), 5987-5991. The results obtained from the above-described computer analysis can be used for, *e.g.*, the preparation of peptidomimetics of the protein of the invention or fragments thereof. Such pseudopeptide analogues of the natural amino acid sequence of the protein may very efficiently mimic the parent protein (Benkirane, *J. Biol. Chem.* 271 (1996), 33218-33224). For example, incorporation of easily available achiral WW-amino acid residues into a protein of the invention or a fragment thereof results in the substitution of amide bonds by polymethylene units of an aliphatic chain, thereby providing a convenient strategy for constructing a peptidomimetic (Banerjee, *Biopolymers* 39 (1996), 769-777). Superactive peptidomimetic analogues of small peptide hormones in other systems are described in the prior art (Zhang, *Biochem. Biophys. Res. Commun.* 224 (1996), 327-331). Appropriate peptidomimetics of the protein of the present invention can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive amide alkylation and testing the resulting compounds, *e.g.*, for their binding

and immunological properties. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715.

Furthermore, a three-dimensional and/or crystallographic structure of the protein of the invention can be used for the design of peptidomimetic inhibitors of the biological activity of the protein of the invention (Rose, Biochemistry 35 (1996), 12933-12944; Rutenber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

"Compound" means any chemical or biological compound, including simple or complex inorganic or organic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates or nucleic acids, that interferes with the interaction of a protein depicted in SEQ ID NO. 2, 4 or 6 with a compound of the CD40 and/or NF-kB related pathway.

As used herein, the term "composition" refers to any composition such as a pharmaceutical composition comprising as an active ingredient a functional protein according to the present invention possibly in the presence of suitable excipients known to the skilled man and may thus be administered in the form of any suitable composition as detailed below by any suitable method of administration within the knowledge of a skilled man. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable excipient. Such excipients are inherently nontoxic and non-therapeutic. Examples of such excipients are saline, Ringer's solution, dextrose solution and Hank's solution. Non-aqueous excipients such as fixed oils and ethyl oleate may also be used. A preferred excipient is 5% dextrose in saline. The excipient may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The functional protein of the invention is administered at a concentration that is therapeutically effective to prevent allograft rejection, GVHD, allergy and autoimmune diseases. The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that the functional protein is given at a dose between 1 mg/kg and 10 mg/kg, more preferably between 10 mg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous short time infusion (during 30



minutes) may also be used. The compositions comprising the functional protein according to the invention may be infused at a dose between 5 and 20 mg/kg/minute, more preferably between 7 and 15 mg/kg/minute.

According to a specific case, the "therapeutically effective amount" of the functional protein according to the invention needed should be determined as being the amount sufficient to cure the patient in need of treatment or at least to partially arrest the disease and its complications. Amounts effective for such use will depend on the severity of the disease and the general state of the patient's health. Single or multiple administrations may be required depending on the dosage and frequency as required and tolerated by the patient.

With regard to the use of the functional protein of the present invention to prevent allograft rejection, it should be stressed that the proteins of the present invention or the compositions comprising the same may be administered before, during or after the organ transplantation as is desired from case to case. In case the protein or the compositions comprising the same are administered directly to the host, treatment will preferably start at the time of the transplantation and continue afterwards in order to prevent the activation and differentiation of host T cells against the MHC on the allograft. In case the donor organ is *ex vivo* perfused with the functional protein according to the invention or the compositions comprising the same, treatment of the donor organ *ex vivo* will start before the time of the transplantation of the donor organ in order to prevent the activation and differentiation of host T cells against the MHC on the allograft.

The invention is further explained by way of the following illustrative examples:

## EXAMPLES

### Example 1: isolation of the CD40 Interacting Proteins

#### Yeast two-hybrid screening.

The two-hybrid assay was performed by the interaction trap cloning method, which is often referred to as the LexA two-hybrid system (Gyuris *et al.*, 1993). The DNA encoding the cytoplasmic part of CD40 (62 amino acids, from residue 216 to 277, where the open reading

frame ends, according to the sequence and numbering as given in Stamenkovic *et al.* (1989)) was generated by PCR and inserted into the EcoRI-SalI digested pEG202 vector (Gyuris *et al.*, 1993), in frame with the LeXA DNA-binding domain (hereinafter the “bait plasmid”). Screening was performed using a HeLa cell fusion library in the plasmid pJG45 (hereinafter the “prey plasmid”), obtained from the laboratory of R. Brent (Harvard Med. School, Boston, MA, USA). Transformation of EGY48 yeast (MAT alpha, *his3*, *trp1*, *ura3-52*, *leu2::pLeu2-LexAop6*) with the prey plasmid, the bait plasmid and the p8op-LacZ (Clontech) reporter plasmid was performed by the Lithium Acetate transformation method (Gietz *et al.*, 1995). The two-hybrid screening was conducted as described in the manual distributed by the laboratory of R. Brent (published in “Gene probes- A practical approach, Oxford University press”).

### Results of the two-hybrid screening.

Yeast containing bait plasmid and lacZ reporter plasmid was transformed with 20 microgram prey library plasmid and plated on glucose medium lacking tryptophan, histidine and uracil, to select for the presence of all three plasmids. In total, approximately  $1.5 \times 10^6$  colonies were obtained. The colonies were harvested and frozen at  $-70^\circ\text{C}$  in a glycerol solution (65% v/v glycerol; 0.1 M  $\text{MgSO}_4$ , 25 mM Tris pH 7.4). From these stocks,  $20 \times 10^6$  colony forming units were plated on galactose medium lacking leucine, tryptophan, histidine and uracil, to screen for protein-protein interaction. Yeast colonies growing on the latter selective medium were further checked for interaction by screening for blue/white staining on medium containing X-gal and galactose. The colonies displaying the following phenotype were picked for further analysis: i) no growth on glucose containing medium which lacks leucine, ii) growth on galactose containing medium lacking leucine, iii) white on medium containing glucose and X-gal, iv) blue on medium containing galactose and X-gal.

Plasmids were isolated from the yeast with the proper phenotype. Analysis of the obtained prey plasmids revealed that the entire screening had finally resulted in the isolation of three different cDNA inserts. Sequencing of the clones showed, in addition to a partial cDNA for TRAF3, two novel cDNA's, termed TTRAP and 4C4.

## Isolation of the full length cDNA

Full length human TTRAP cDNA was obtained by screening a HUVEC cDNA library with the TTRAP fragment as probe. A cDNA of about 2 kb was isolated, with an open reading frame of 1086 nucleotides encoding for a protein of 362 amino acids (SEQ ID NO.2).

5 The mouse TTRAP homologue was obtained by screening the EST database and aligning the homologous sequences. Human and mouse TTRAP are approximately 65% identical and 70% similar on the amino acid level. The mouse sequence is shown in SEQ ID NO. 3.

## Example 2: Sequence Analysis of the cDNA's

10 Nucleotide sequence analysis was carried out using dye terminator mix and a 310 Genetic analyzer from Perkin Elmer. The nucleotide sequence of TTRAP is shown in SEQ ID NO.1 whereas the sequence of 4C4 is shown in SEQ ID NO.5.

The TTRAP sequence shows a low homology (30% similarity at amino acid level) with Nocturnin, a protein that is expressed in the photoreceptor of the eye of *Xenopus laevis* (Green and Beshare, 1996). The partial sequence of the mouse homologue of Nocturnin is known (Puech  
15 *et al.*, 1997). Additionally, there is some homology with EST sequences (e.g., Genbank EST c23016, aa162513, aa571061, t87026, h45114, aa196281, h94108 and aa337396) and with the C-terminal part of the yeast transcription factor *CCR4* (Malvar *et al.*, 1992). Although these homologies are low it is not excluded that a human homologue of these proteins would bind to the cytoplasmic domain of CD40.

20 It is interesting to note that, unexpectedly, TTRAP neither 4C4 show any significant homology with TRAF's or other proteins known to interact with CD40.

## Example 3: Study of the Interaction of TTRAP Protein, 4c4 Protein and TTRAP Protein Fragments with Other Proteins Using a Yeast Two-hybrid Interaction Assay

25 The potential binding of TTRAP to other proteins was assessed using a yeast two-hybrid assay. The experimental outline is similar to the one described for the two-hybrid screening. However, here the plasmids for bait, prey and lacZ reporter were transformed simultaneously into the EGY48 yeast strain. Positive interaction was determined either by the growth phenotype

(growth on medium lacking leucine in the presence of galactose, and not in the presence of glucose) or by the blue/white staining on X-gal containing plates (blue colonies only on galactose containing plates, not on glucose containing plates). cDNA's for TRAF2 and for the cytoplasmic regions of CD30, CD40 and TNF-RII were generated by PCR using the pfu polymerase (Promega). PCR fragments encoding RIP, TRADD and FADD were cloned in pCDNA3 (Invitrogen, Carlsbad, CA). cDNA of TRAF3 was obtained from the laboratory of Dixit, Dept Pathol., Univ. Michigan Med. School, MI, USA). The color formation was evaluated as strong and fast (++), strong but slow (+), weak and slow (+/-), none (-) or not determined (nd)

The results for TTRAP protein and TTRAP fragments used are summarized in Table I and FIG. 1 respectively.

**Table I**

	TTRAP	4F2	4F2d3	4F2d2	4F2d1	4C4	---
<b>CD40</b>	++	++	+/-	+/-	+/-	+	-
<b>CD30</b>	++	++	+/-	+/-	+/-	+	-
<b>TNF-RII</b>	+	+	-	-	-	+	-
<b>LMP-1</b>	-	-	nd	nd	nd	-	-
<b>TRAF2</b>	+	+	nd	nd	nd	nd	-
<b>TRAF3</b>	+	+	+/-	+/-	+/-	nd	-
<b>RIP</b>	++	++	+/-	+/-	+/-	nd	+/-
<b>TRADD</b>	+	nd	nd	nd	nd	nd	-
<b>FADD</b>	-	nd	nd	nd	nd	nd	-
<b>4F2</b>	++	++	-	-	-	+	-
<b>4C4</b>	++	++	-	-	-	+	-

TTRAP, as well as the longest TTRAP fragment (aa 54 - 362) shows a strong interaction with CD40, CD30, RIP and with 4C4, and a weaker interaction with TNF-RII and TRAF3.

Remarkably, TTRAP can also self-associate. TTRAP fragments, missing the C-terminal end (aa 274 - 362) show only a weak interaction.

Yeast transformed with TTRAP or 4F2 as a prey, and TRAF2 as a bait, gives a positive interaction phenotype, similar to the one observed for TTRAP and TRAF3. This clearly indicates that TTRAP/4F2 also associates with TRAF2.

Because of the interaction of TTRAP with TRAF2 and 3 in two-hybrid assays in yeast, and with TRAF5 in co-immunoprecipitations from mammalian cells (see example 6) the conclusion is justified that TTRAP binds all other TRAFs as well. Deletion mutants of TRAFs are constructed to find out whether the region commonly denominated as the TRAF domain is responsible for the binding of the TRAFs to TTRAP. In a similar approach deletion mutants of TTRAP are constructed to find out which region in the protein is required and sufficient for the interaction with TRAFs.

4C4 protein is interacting with CD40, CD30, TNF-RII, with the longest fragment of TTRAP and with a deletion mutant of TRAF3 which still contains the largest part of the TRAF domain (from aa 380 to the carboxy terminal end of the protein. A smaller form of 4C4 (from amino acid 2 - amino acid 245 in SEQ ID NO.6) is also capable to interact with CD40.

#### **Example 4: Expression Pattern of TTRAP and 4C4**

The TTRAP gene is widely expressed, as was already indicated by the presence of several partial TTRAP cDNA's in the EST sequence data base. The TTRAP expression was analyzed by Northern blot analysis against mRNA from different tissues, both from human and mouse (FIG. 2). Human TTRAP is present as a 2.2 kb transcript in all tissues tested. In addition to the 2.2 kb transcript an additional 1.7 kb transcript is present in a testis sample. (FIG. 2A).

Human TTRAP expression was further tested and found in the B-cell lines BJAB (Menezes *et al.*, 1975) and DG75 (Ben-Bassat *et al.*, 1977), in the Jurkat T-cell line and in HUVECs (Human umbilical vein endothelial cell).

For mouse TTRAP, two transcripts, one of 2.2 kb and one of 3.8 kb was found on a murine multiple Northern blot (FIG. 2B). Mouse TTRAP mRNA was also detected in all tissues tested, be it to a lower extent in skeletal muscle. Both mouse transcripts are not only present in

adult animals, but can also be detected in mouse embryo's from 7 to 17 days post coitus. These results are an indication that TTRAP plays an important role in early development.

On a multiple tissue Northern blot, a 4C4 probe recognizes 3 transcripts, of 1.6kb, 3.5 kb and 7.5kb. All three mRNA's are present in spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes. The expression of the 3.5 kb transcript is most prominent in testis. In ovary, the signal of the 7.5 kb mRNA is strongest.

### **Example 5: Co-immunoprecipitation of TTRAP with TNF-RII**

It has been shown by two-hybrid analysis in yeast that TTRAP interacts with different receptors of the TNF-receptor superfamily, i.e. CD30, CD40 and TNF-RII (see, Table I). To confirm these results for one of these receptors in mammalian cells, co-immunoprecipitation of TTRAP with TNF-RII was carried out. In a typical experiment, 293T cells were transfected with HA-tagged TTRAP and TNF-RII (both in pCDNA expression vectors). TNF-RII was immunoprecipitated with the utr4 monoclonal antibody (gift from Roche, Basel). By Western blot on the immunoprecipitated fraction it was confirmed that TTRAP interacts with TNF-RII.

The method used hereto is as follows: A 9cm dish with 293T Human Embryonic Kidney cells (approx. 30-50% confluent) is transfected with approximately 2 microgram of each expression plasmid. 24-48 hours after transfection, the cells are harvested and lysed in 300 microliter lysis buffer (50mM Tris/HCl pH 7.4, 200mM NaCl, 10% glycerol, 0.2% NP-40, 50mM NaF, 1 mM  $\text{Na}_3\text{P}_2\text{O}_7$ , 1mM  $\text{Na}_3\text{VO}_4$  and protease inhibitors). The cell suspension is incubated for 20 minutes on ice. Cellular debris is pelleted for 10 minutes in an Eppendorf centrifuge, at 14.000 rpm and 4°C, and the supernatant is transferred into a fresh tube. 5 microgram antibody is added to the lysate, and incubated for 3 hours at 4°C, on a mixing platform. The samples are supplemented with 20 microliter of a 50% slurry of protein A or G beads and the incubation is continued for 1 hour. The beads are pelleted for 1 minute at minimal speed (approx. 500 rpm) and the supernatant is removed. The beads are resuspended in 750 microliter lysis buffer and immediately centrifuged again. This washing procedure is repeated, but this time samples are incubated for 10-15 minutes at 4°C on a mixing platform. This is repeated twice more, to come to a total of 4 buffer changes. After the last wash, as much buffer as possible is to be removed, without sucking up Sepharose beads. After addition of 20 microliter

sample buffer, the samples are stored at  $-20^{\circ}\text{C}$  or processed for SDS/PAGE. 10 microliter of each sample is analyzed to determine the protein CoIP, and 3 microliter to verify the IP.

Interestingly, it was found that the association of TTRAP with the TNF receptor in an immunoprecipitation protocol from mammalian cells, is preserved only if the immunoprecipitation procedure was performed in the presence of inhibitors of protein phosphatases, i.e. 50mM NaF, 1 mM  $\text{Na}_3\text{P}_2\text{O}_7$ , 1 mM  $\text{Na}_3\text{VO}_4$ . This suggests that TTRAP and/or the receptor are phosphorylated, and that this phosphorylation is required for the interaction of both proteins.

#### **Example 6: Interaction of TTRAP with TRAF3 and TRAF5**

TTRAP was initially isolated in a two-hybrid screen in yeast, using the cytoplasmic domain of CD40 as bait. The interaction of TTRAP with CD40 could subsequently be confirmed by co-immunoprecipitation of both proteins, after over-expression in 293T human embryonic kidney cells. Because CD40 is known to associate with TRAFs in mammalian cells, it was investigated whether TTRAP could bind to TRAF3 and TRAF5.

The interaction of TTRAP with TRAF3 is disclosed in Table I. To investigate whether TTRAP could interact with TRAF5, a co-immunoprecipitation experiment was performed.

In this experiment immunoprecipitation was carried out for Flag-tagged human TRAF5 (gift from Jun-ichiro Inoue, Dept Oncology, Inst. Med Sci., Univ. Tokyo, Japan) from cells co-transfected with TRAF5 and TTRAP. Co-immunoprecipitation of HA-tagged TTRAP was detected by analysing the immunoprecipitated proteins on Western blots. These results clearly demonstrate that TTRAP forms a complex with TRAF5 in mammalian cells.

#### **Example 7: Interaction of TTRAP with CD40**

To delineate the region in the cytoplasmic tail of CD40 that is important for TTRAP binding, deletion mutants of the receptor are made and are tested in co-immunoprecipitations and yeast two-hybrid assays. In a similar approach, deletion mutants of TTRAP are made to find out which region in the protein is necessary and sufficient for the interaction with CD40.

#### **Example 8: Genomic TTRAP Sequence**

Recently, the human genomic sequence for TTRAP has been deposited to the EMBL database (accession number HS30M3). The chromosome location of the genomic clone was mapped to 6p22.1-22.3. The TTRAP sequence within this clone was identified as a novel protein, termed dJ30M3.3 (acc number, CAA21141). The mRNA sequence starts at nucleotide 47,151 and ends at position 64,053. The protein encoding region starts at 47,168 and stops at 63,242, comprising 7 exons. The translational start and stop sites match those that were determined for TTRAP by cDNA library screening and the amino acid sequence is identical to the human sequence as disclosed herein.

The human TTRAP sequence also aligns with the *C. elegans* gene product Y63D3A.4 (accession number CEY63D3A\_4). At the protein level, the sequences are approximately 30% identical. On the basis of the protein alignment it is assumed that this *C. elegans* protein is the nematode homologue of human TTRAP.

### Example 9: Identification of Novel CD40-interacting Proteins

A two-hybrid screening in yeast was performed with the cytoplasmic tail of human CD40 as a bait, and a HeLa cDNA library as a prey. In this screening, the proteins encoded by the cDNAs listed below, were picked up as a result of their interaction with CD40.

1. A partial cDNA encoding human Bloom's syndrome protein (BLM) (accession number U39817). The complete cDNA sequence of BLM is 4,437 nucleotides. The protein encoding sequence starts at nucleotide 75 and stops at nucleotide 4,328. The size of the protein is 1,418 amino acids. The partial cDNA that was picked up in the two-hybrid screen with CD40 starts at nucleotide 529, which corresponds to amino acid 151. The 3'-end of this partial cDNA clone has not been determined yet.
2. A partial cDNA encoding for human nuclear autoantigen (SP-100) (accession number M60618). The complete cDNA sequence of SP-100 is 1,879 nucleotides. The protein encoding sequence starts at nucleotide 32 and stops at nucleotide 1,474. The size of the protein is 481 amino acids. The partial cDNA that was picked up in the two-hybrid screen with CD40 starts at nucleotide 699, which corresponds to amino acid 223, and reaches till the end of the SP-100 cDNA sequence.



3. A partial cDNA sequence for the human homologue of the mouse BP75 protein (accession number AF084259). The complete cDNA sequence of the mouse protein BP75 is 2,361 nucleotides. The protein encoding sequence starts at nucleotide 211 and stops at nucleotide 2,166. The size of the protein is 652 amino acids. The partial cDNA that was picked up in the two-hybrid screen with CD40, aligns with the mouse cDNA sequence starting at nucleotide 1,070, which corresponds to amino acid 286 , and goes to the end of the BP75 cDNA sequence. The mouse and the human cDNA sequences are 83% identical. The human genomic sequence of BP75 has also been deposited to the database (accession number Z99496). The cDNA of mouse BP75 is approximately 83% identical to the human genomic PAC clone (from nucleotide 122564-120820). The chromosome location of the human PAC clone was mapped to 6q22.1-22.33.

#### **Example 10: Isolation of DAXX as a CD40-interacting Protein by Yeast two-hybrid**

In a yeast two-hybrid assay with the cytoplasmic tail of human CD40 as a bait, a partial cDNA was picked up encoding the human Fas-binding protein (DAXX) (accession number AF039136). The complete cDNA sequence of DAXX is 2,487 nucleotides. The protein encoding sequence starts at nucleotide 148 and stops at nucleotide 2,370. The size of the protein is 741 amino acids. The partial cDNA that was picked up in the two-hybrid screen with CD40 starts at nucleotide 1,500, which corresponds to amino acid 451, and goes to the end of the DAXX cDNA sequence.

Fas and CD40 are both members of the TNF-Receptor superfamily. DAXX was originally isolated as a Fas-binding protein, in a yeast two-hybrid screen (Yang et al., Cell, 89, 1067-76, 1997). The protein was shown to interact specifically with the death domain of Fas. It was reported to play a role in apoptosis via the activation of the Jun N-terminal kinase. The authors examined the binding of a partial clone of human DAXX (from amino acid 501 till the end) to the cytoplasmic tail of mouse CD40, and could not detect interaction. In addition, an *in vitro* interaction assay of full length DAXX with GST-CD40 also turned out to be negative. Therefore, the authors conclude that DAXX does not associate with CD40.

The discrepancy between some of the findings reported in Yang et al. and the observation according to the current invention that the cytoplasmic tail of CD40 does interact

with DAXX in yeast two-hybrid, could be due to the following reasons. i) In the two-hybrid interaction assay that was performed, Yang et al. use a shorter partial clone of DAXX than the one picked up according to the present invention. In addition interaction of a partial human DAXX with human CD40 is observed in the underlying invention, whereas Yang et al., use a partial human DAXX and mouse CD40. ii) The *in vitro* binding assay that Yang et al use, may not be sensitive enough to detect the interaction of full-length DAXX and CD40 protein. Moreover, it is unclear whether in this assay Yang et al use both proteins from the same species.

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# SEQUENCE LISTING

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(H) TELEFAX: +32 9 2446610

(ii) TITLE OF INVENTION: CD40 interacting and TRAF interacting proteins

(iii) NUMBER OF SEQUENCES: 6

### (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

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(A) LENGTH: 1920 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

### (vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

### (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 20..1108

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  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
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(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Ser Gln Glu Ile Ile Pro Phe Pro Ser Thr Lys Met Met Arg Asn Leu  
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Ser His Leu Glu Ser Thr Arg Gly His Ala Ala Glu Arg Met Asn Gln  
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Val Ile Phe Ala Gly Asp Thr Asn Leu Arg Asp Arg Glu Val Thr Arg  
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Cys Gly Gly Leu Pro Asn Asn Ile Val Asp Val Trp Glu Phe Leu Gly  
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40 Lys Pro Lys His Cys Gln Tyr Thr Trp Asp Thr Gln Met Asn Ser Asn  
 290 295 300

Leu Gly Ile Thr Ala Ala Cys Lys Leu Arg Phe Asp Arg Ile Phe Phe

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|   | Arg Ala Ala Ala         | Glu Gly His Ile     | Ile Pro Arg Ser | Leu Asp Leu |
|   |                         | 325                 | 330             | 335         |
| 5 | Leu Gly Leu Glu Lys Leu | Asp Cys Gly Arg Phe | Pro Ser Asp His | Trp         |
|   |                         | 340                 | 345             | 350         |
|   | Gly Leu Leu Cys Asn Leu | Asp Ile Ile Leu     |                 |             |
|   |                         | 355                 | 360             |             |

(2) INFORMATION FOR SEQ ID NO: 3:

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 (A) LENGTH: 1312 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Mus musculus

20 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION:122..1234

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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| 35 | Gly Cys Asp Pro Thr Met Val Pro Ser Val Leu Arg Glu Asn Asp Trp                                                              |     |
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|    | Gln Thr Gln Lys Ala Leu Ser Ala Tyr Phe Glu Leu Pro Glu Asn Asp                                                              |     |
|    | 65 70 75                                                                                                                     |     |
| 40 | CAA GGG TGG CCG CGC CAG CCT CCC ACG TCC TTC AAG TCC GAG GCC TAT                                                              | 406 |
|    | Gln Gly Trp Pro Arg Gln Pro Pro Thr Ser Phe Lys Ser Glu Ala Tyr                                                              |     |
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|    |  |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |      |  |
|----|--|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|------|--|
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| 20 |  | CCT<br>Pro        | TTT<br>Phe<br>210 | CCA<br>Pro        | AAT<br>Asn        | ACC<br>Thr        | AAA<br>Lys        | ATG<br>Met        | ATG<br>Met<br>215 | AGA<br>Arg        | AAC<br>Asn        | CTG<br>Leu        | CTA<br>Leu<br>220 | TGC<br>Cys        | GTA<br>Val        | AAT<br>Asn        | GTG<br>Val        | 790  |  |
|    |  | AGT<br>Ser        | TTG<br>Leu<br>225 | GGT<br>Gly        | GGA<br>Gly        | AAT<br>Asn        | GAA<br>Glu        | TTT<br>Phe<br>230 | TGC<br>Cys        | CTT<br>Leu        | ATG<br>Met        | ACA<br>Thr        | TCC<br>Ser<br>235 | CAT<br>His        | TTG<br>Leu        | GAG<br>Glu        | AGC<br>Ser        | 838  |  |
| 25 |  | ACC<br>Thr<br>240 | AGA<br>Arg        | GAA<br>Glu        | CAT<br>His        | TCT<br>Ser        | GCG<br>Ala<br>245 | GAA<br>Glu        | CGA<br>Arg        | ATA<br>Ile        | AGA<br>Arg        | CAA<br>Gln<br>250 | TTA<br>Leu        | AAA<br>Lys        | ACT<br>Thr        | GTT<br>Val        | CTT<br>Leu<br>255 | 886  |  |
|    |  | GGA<br>Gly        | AAA<br>Lys        | ATG<br>Met        | CAA<br>Gln<br>260 | GAG<br>Glu        | GCT<br>Ala        | CCA<br>Pro        | GAT<br>Asp        | TCA<br>Ser        | ACC<br>Thr<br>265 | ACG<br>Thr        | GTT<br>Val        | ATA<br>Ile        | TTT<br>Phe        | GCA<br>Ala<br>270 | GGA<br>Gly        | 934  |  |
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| 35 |  | GAC<br>Asp        | AAC<br>Asn<br>290 | GTT<br>Val        | TTT<br>Phe        | GAT<br>Asp        | GCC<br>Ala        | TGG<br>Trp        | GAA<br>Glu<br>295 | TTT<br>Phe        | TTA<br>Leu        | GGC<br>Gly        | AAA<br>Lys        | CCT<br>Pro<br>300 | AAA<br>Lys        | CAT<br>His        | TGC<br>Cys        | 1030 |  |
|    |  | CAG<br>Gln        | TAT<br>Tyr<br>305 | ACA<br>Thr        | TGG<br>Trp        | GAT<br>Asp        | ACG<br>Thr        | AAA<br>Lys<br>310 | GCA<br>Ala        | AAT<br>Asn        | AAC<br>Asn        | AAC<br>Asn        | CTC<br>Leu<br>315 | AGG<br>Arg        | ATC<br>Ile        | CCT<br>Pro        | GCT<br>Ala        | 1078 |  |
| 40 |  | GCT<br>Ala<br>320 | TAT<br>Tyr        | AAG<br>Lys        | CAT<br>His        | CGT<br>Arg        | TTT<br>Phe<br>325 | GAT<br>Asp        | CGA<br>Arg        | ATA<br>Ile        | TTT<br>Phe        | TTC<br>Phe<br>330 | AGA<br>Arg        | GCA<br>Ala        | GAA<br>Glu        | GAG<br>Glu        | GGG<br>Gly<br>335 | 1126 |  |
|    |  | CAC<br>His        | CTT<br>Leu        | ATT<br>Ile        | CCT<br>Pro<br>340 | CAA<br>Gln        | AGT<br>Ser        | TTA<br>Leu        | GAC<br>Asp        | CTT<br>Leu<br>345 | GTT<br>Val        | GGG<br>Gly<br>350 | TTG<br>Leu        | GAA<br>Glu        | AAA<br>Lys        | CTG<br>Leu        | GAC<br>Asp        | 1174 |  |
| 45 |  | TGT<br>Cys        | GGT<br>Gly        | AGA<br>Arg<br>355 | TTT<br>Phe        | CCG<br>Pro        | AGT<br>Ser        | GAT<br>Asp        | CAC<br>His        | TGG<br>Trp<br>360 | GGG<br>Gly        | CTC<br>Leu        | TTG<br>Leu        | TGC<br>Cys        | ACC<br>Thr        | TTG<br>Leu        | AAT<br>Asn        | 1222 |  |

GTA GTA TTG TGA AAAGCTTCCC ACTTGCAGCT TTACACGTTT GTTAGCACTA  
 Val Val Leu \*  
 370

1274

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1312

5 (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 371 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

- 10 (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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 35 40 45

Cys Asp Pro Thr Met Val Pro Ser Val Leu Arg Glu Asn Asp Trp Gln  
 50 55 60

20 Thr Gln Lys Ala Leu Ser Ala Tyr Phe Glu Leu Pro Glu Asn Asp Gln  
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Gly Trp Pro Arg Gln Pro Pro Thr Ser Phe Lys Ser Glu Ala Tyr Val  
 85 90 95

25 Asp Leu Thr Asn Glu Asp Ala Asn Asp Thr Thr Ile Leu Glu Ala Ser  
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 145 150 155 160

Gln Glu Val Ile Pro Pro Tyr Cys Ala Tyr Leu Lys Lys Arg Ala Ala  
 165 170 175

35 Ser Tyr Thr Ile Ile Thr Gly Asn Glu Glu Gly Tyr Phe Thr Ala Ile  
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Leu Leu Lys Lys Gly Arg Val Lys Phe Lys Ser Gln Glu Ile Ile Pro  
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Phe Pro Asn Thr Lys Met Met Arg Asn Leu Leu Cys Val Asn Val Ser  
 210 215 220

40 Leu Gly Gly Asn Glu Phe Cys Leu Met Thr Ser His Leu Glu Ser Thr  
 225 230 235 240

Arg Glu His Ser Ala Glu Arg Ile Arg Gln Leu Lys Thr Val Leu Gly  
 245 250 255



```

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ATTACAGGAA AAAAATGGAG AGGTAAATTG TTTAAATACA CGTTACAGTG CAACAAGAAT     1080
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TGCCAAAATC TGTATCATAA TTGATGAGGC ATCTACAGTT TCAAAGAAAA CCACCCTAGT     1200
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15 TGCTAATACA ANCCTGGGAA GAAAGTCTGG AGTAGCTACA AAATTGTTAG AAAATTTTCC     1440
TGAAATCATC ATTTGGAAC TTTTAAATCA TCGATTACAA TTGTCACCTG ATGATTCTAT     1500
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(2) INFORMATION FOR SEQ ID NO: 6:

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20  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 442 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

25  (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO

      (vi) ORIGINAL SOURCE:
          (A) ORGANISM: homo sapiens

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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35  Phe Val Ser Glu Gly Pro Ser Arg Pro Val Leu Glu Tyr Ile Asp Leu
     35          40          45

     Val Cys Gly Asp Asp Glu Asn Pro Ser Ala Tyr Tyr Ser Asp Ile Leu

```

|    | 50                                                                                 | 55 | 60 |
|----|------------------------------------------------------------------------------------|----|----|
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| 5  | Met Lys Lys Val Lys Thr Asp Thr Glu Asn Asn Glu Val Ser Lys Asn<br>85 90 95        |    |    |
|    | His Cys Arg Leu Ser Lys Ala Lys Glu Pro His Phe Glu Tyr Ile Glu<br>100 105 110     |    |    |
|    | Gln Pro Ile Ile Glu Glu Lys Pro Ser Leu Ser Ser Lys Lys Glu Ile<br>115 120 125     |    |    |
| 10 | Asp Asn Leu Val Leu Pro Asp Cys Trp Asn Glu Lys Gln Ala Phe Met<br>130 135 140     |    |    |
|    | Phe Thr Glu Gln Tyr Lys Trp Leu Glu Ile Lys Glu Gly Lys Leu Gly<br>145 150 155 160 |    |    |
| 15 | Cys Lys Asp Cys Ser Ala Val Arg His Leu Gly Ser Lys Ala Glu Lys<br>165 170 175     |    |    |
|    | His Val His Val Ser Lys Glu Trp Ile Ala Tyr Leu Val Thr Pro Asn<br>180 185 190     |    |    |
|    | Gly Ser Asn Lys Thr Thr Arg Gln Ala Ser Leu Arg Lys Lys Ile Arg<br>195 200 205     |    |    |
| 20 | Glu His Asp Val Ser Lys Ala His Gly Lys Ile Gln Asp Leu Leu Lys<br>210 215 220     |    |    |
|    | Glu Ser Thr Asn Asp Ser Ile Cys Asn Leu Val His Lys Gln Asn Asn<br>225 230 235 240 |    |    |
| 25 | Lys Asn Ile Asp Ala Thr Val Lys Val Phe Asn Thr Val Tyr Ser Leu<br>245 250 255     |    |    |
|    | Val Lys His Asn Arg Pro Leu Ser Asp Ile Glu Gly Ala Arg Glu Leu<br>260 265 270     |    |    |
|    | Gln Glu Lys Asn Gly Glu Val Asn Cys Leu Asn Thr Arg Tyr Ser Ala<br>275 280 285     |    |    |
| 30 | Thr Arg Ile Ala Glu His Ile Ala Lys Glu Met Lys Met Lys Ile Phe<br>290 295 300     |    |    |
|    | Lys Asn Ile Ile Glu Glu Asn Ala Lys Ile Cys Ile Ile Ile Asp Glu<br>305 310 315 320 |    |    |
| 35 | Ala Ser Thr Val Ser Lys Lys Thr Thr Leu Val Ile Tyr Leu Gln Cys<br>325 330 335     |    |    |
|    | Thr Ile Gln Ser Ala Pro Ala Pro Val Met Leu Phe Val Ala Leu Lys<br>340 345 350     |    |    |
|    | Glu Leu Val Ser Thr Ile Ala Glu Cys Ile Val Asn Thr Leu Leu Thr<br>355 360 365     |    |    |
| 40 | Thr Leu Asn Asp Cys Gly Phe Thr Asn Glu Tyr Leu Lys Ala Asn Leu<br>370 375 380     |    |    |
|    | Ile Ala Phe Cys Ser Asp Gly Ala Asn Thr Xaa Leu Gly Arg Lys Ser<br>385 390 395 400 |    |    |

|   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|   | Gly | Val | Ala | Thr | Lys | Leu | Leu | Glu | Asn | Phe | Pro | Glu | Ile | Ile | Ile | Trp |
|   |     |     |     |     | 405 |     |     |     |     | 410 |     |     |     |     | 415 |     |
|   | Asn | Cys | Leu | Asn | His | Arg | Leu | Gln | Leu | Ser | Leu | Asp | Asp | Ser | Ile | Ser |
|   |     |     |     | 420 |     |     |     |     | 425 |     |     |     |     | 430 |     |     |
| 5 | Glu | Ile | Lys | Gln | Ile | Asn | His | Leu | Xaa | Tyr |     |     |     |     |     |     |
|   |     |     | 435 |     |     |     |     | 440 |     |     |     |     |     |     |     |     |

## CLAIMS

What is claimed is:

1. An isolated functional protein capable of interacting with the cytoplasmic domain of CD40  
and/or other receptors of the TNF receptor superfamily such as CD30 and TNF receptor II  
5 wherein the protein has no homology to TRAF-proteins.
2. An isolated functional protein according to claim 1 comprising an amino acid sequence with  
70-100% homology to the amino acid sequence depicted in SEQ ID NO. 2.
3. An isolated functional protein according to claim 1 comprising an amino acid sequence with  
70-100% homology to the amino acid sequence depicted in SEQ ID NO. 4.
- 10 4. An isolated functional protein according to claim 1 comprising an amino acid sequence with  
70-100% homology to the amino acid sequence depicted in SEQ ID NO. 6.
5. An isolated functional protein according to claim 1 comprising an amino acid sequence with  
70-100% homology to the amino acids 54-362 of SEQ ID NO. 2.
6. An isolated functional protein according to claim 5 comprising an amino acid sequence with  
15 70-100% homology to the amino acids 274-362 of SEQ ID NO. 2.
7. An isolated functional protein according to claim 1 comprising an amino acid sequence with  
70-100% homology to the amino acids 2-245 of SEQ ID NO.6.
8. A nucleic acid sequence encoding a protein according to any of the claims 1-7.
9. A nucleic acid sequence according to claim 8 with 70-100% homology to the DNA sequence  
20 depicted in SEQ ID NO. 1.
10. A nucleic acid sequence according to claim 8 with 70-100% homology to the DNA  
sequence depicted in SEQ ID NO.3.

11. A nucleic acid sequence according to claim 8 with 70-100% homology to the DNA sequence depicted in SEQ ID NO. 5.
12. Use of an isolated functional protein and/or a functional fragment thereof according to any of the claims 1-7 as a medicament.
- 5 13. Use of an isolated functional protein according to any of the claims 1-7 and/or a functional fragment thereof to treat TRAF-related, CD40-related, NF-kB related and/or Jun (kinase)-related diseases.
14. The use according to claim 13 in which the disease is atherosclerosis, arthritis, multiple sclerosis, systemic lupus erythematosus and/or graft rejection.
- 10 15. The use of an isolated functional protein according to any of the claims 1-7 and/or a functional fragment thereof to sensitize tumor cells to anti-tumor treatments.
16. The use of an isolated functional protein according to any of the claims 1-7 and/or a functional fragment thereof to screen for compounds that interfere with the interaction of said protein(s) with other protein components of the TRAF, CD40 or NF-kB related pathway.
- 15 17. A method for screening compounds comprising the use of a protein according to claim 16.
18. A compound isolated with the method according to claim 17.
19. A pharmaceutical composition comprising one or more isolated functional proteins according to any of the claims 1-7 and/or functional fragments thereof and a pharmaceutical acceptable carrier material.
- 20 20. A pharmaceutical composition comprising one or more compounds according to claim 18 and a pharmaceutical acceptable carrier material.

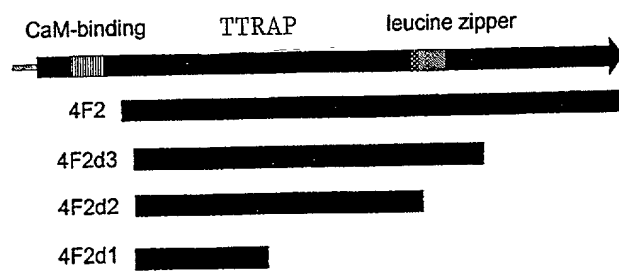


21. Use of a protein according to any of the claims 1-7 and/or functional fragments thereof for the manufacture of a pharmaceutical composition to treat TRAF, CD40 and/or NF-kB related diseases.

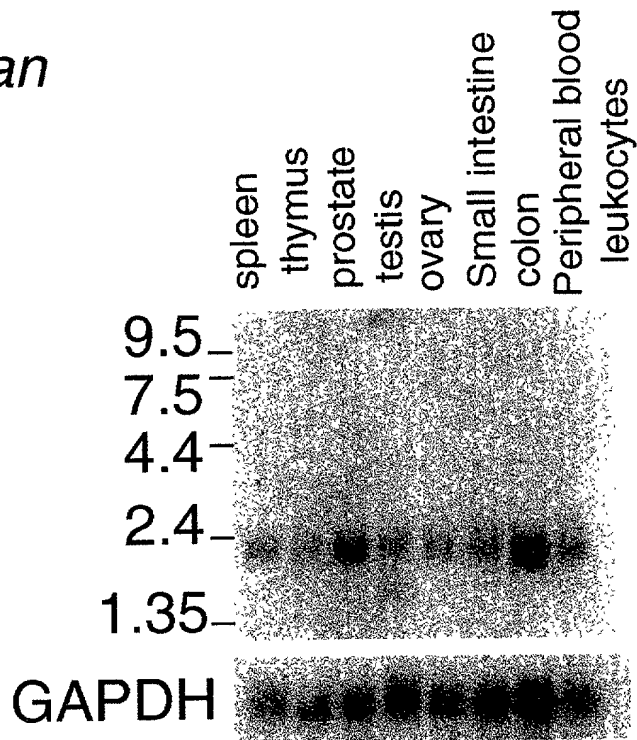
## Abstract

The present invention relates to novel proteins interacting with the cytoplasmic domain of CD40, which are useful in the treatment of CD40 and/or NF- $\kappa$ B related diseases. Surprisingly, these proteins do not show significant homology with the TRAF-protein family and therefore offer the possibility to modulate the CD40 and/or NF- $\kappa$ B pathway independently from the TRAF-CD40 interaction.

Fig. 1



*Human*



*Mouse*

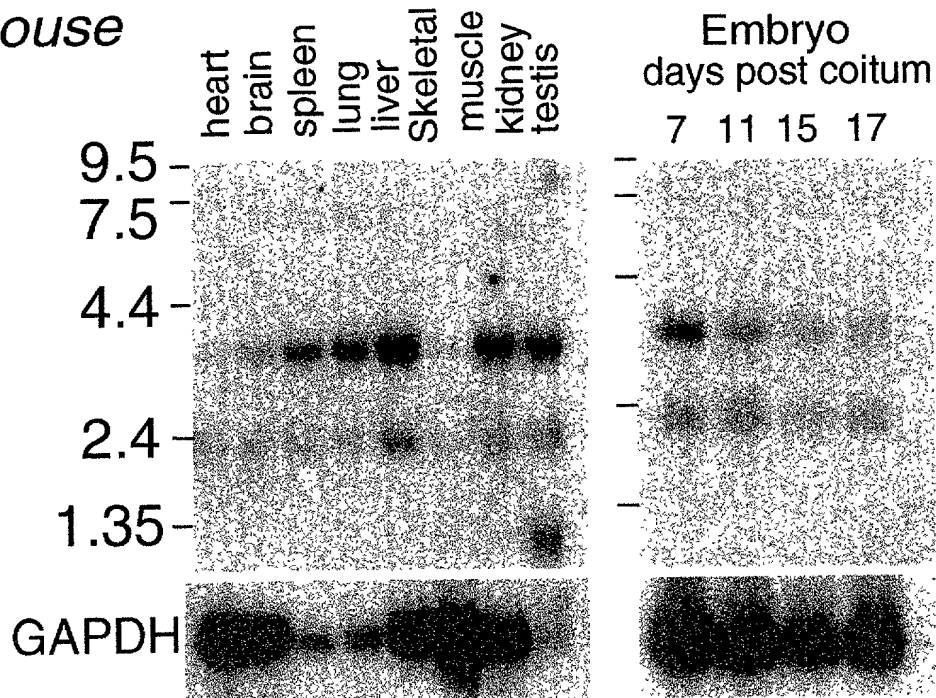


Figure 2

# SEQUENCE LISTING

<110> VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOL

<120> CD40-INTERACTING AND TRAF-INTERACTING PROTEINS

<130> V7/002-V018

<140> PCT/EP99/03025

<141> 1999-04-28

<150> 98201392.2

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<170> PatentIn Ver. 2.1

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Glu Thr Ile Ser Glu Pro Lys Thr Tyr Val Asp Leu Thr Asn Glu Glu
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Thr Thr Asp Ser Thr Thr Ser Lys Ile Ser Pro Ser Glu Asp Thr Gln
          95                100                105

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002207 19870505

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Thr Gln Lys Ala Leu Ser Ala Tyr Phe Glu Leu Pro Glu Asn Asp Gln
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|                                                                 |      |
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| Asn Leu Val His Lys Gln Asn Asn Lys Asn Ile Asp Ala Thr Val Lys |      |
| 235 240 245                                                     |      |
| gtt ttc aat act gtt tac agt tta gta aaa cat aac aga cct tta tct | 1000 |
| Val Phe Asn Thr Val Tyr Ser Leu Val Lys His Asn Arg Pro Leu Ser |      |
| 250 255 260                                                     |      |
| gat att gag ggg gca aga gaa tta cag gaa aaa aat gga gag gta aat | 1048 |
| Asp Ile Glu Gly Ala Arg Glu Leu Gln Glu Lys Asn Gly Glu Val Asn |      |
| 265 270 275 280                                                 |      |
| tgt tta aat aca cgt tac agt gca aca aga ata gca gaa cat att gca | 1096 |
| Cys Leu Asn Thr Arg Tyr Ser Ala Thr Arg Ile Ala Glu His Ile Ala |      |
| 285 290 295                                                     |      |
| aaa gaa atg aag atg aag ata ttt aag aat att ata gaa gag aat gcc | 1144 |
| Lys Glu Met Lys Met Lys Ile Phe Lys Asn Ile Ile Glu Glu Asn Ala |      |
| 300 305 310                                                     |      |
| aaa atc tgt atc ata att gat gag gca tct aca gtt tca aag aaa acc | 1192 |
| Lys Ile Cys Ile Ile Ile Asp Glu Ala Ser Thr Val Ser Lys Lys Thr |      |
| 315 320 325                                                     |      |
| acc cta gtg att tat ctc cag tgc aca att cag tca gct cct gca cct | 1240 |
| Thr Leu Val Ile Tyr Leu Gln Cys Thr Ile Gln Ser Ala Pro Ala Pro |      |
| 330 335 340                                                     |      |
| gtt atg tta ttt gtg gct tta aaa gaa ttg gtg tca act ata gca gag | 1288 |
| Val Met Leu Phe Val Ala Leu Lys Glu Leu Val Ser Thr Ile Ala Glu |      |
| 345 350 355 360                                                 |      |
| tgt att gtc aat aca tta ttg act act tta aat gat tgt ggt ttt aca | 1336 |
| Cys Ile Val Asn Thr Leu Leu Thr Thr Leu Asn Asp Cys Gly Phe Thr |      |
| 365 370 375                                                     |      |
| aat gaa tat ttg aaa gca aat tta att gca ttt tgt tct gat ggt gct | 1384 |
| Asn Glu Tyr Leu Lys Ala Asn Leu Ile Ala Phe Cys Ser Asp Gly Ala |      |
| 380 385 390                                                     |      |
| aat aca anc ctg gga aga aag tct gga gta gct aca aaa ttg tta gaa | 1432 |
| Asn Thr Xaa Leu Gly Arg Lys Ser Gly Val Ala Thr Lys Leu Leu Glu |      |
| 395 400 405                                                     |      |
| aat ttt cct gaa atc atc att tgg aac tgt tta aat cat cga tta caa | 1480 |

Asn Phe Pro Glu Ile Ile Ile Trp Asn Cys Leu Asn His Arg Leu Gln  
 410 415 420

ttg tca ctt gat gat tct ata tcc gaa ata aaa caa att aat cat tta 1528  
 Leu Ser Leu Asp Asp Ser Ile Ser Glu Ile Lys Gln Ile Asn His Leu  
 425 430 435 440

ann tat aa 1536  
 Xaa Tyr

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 <212> PRT  
 <213> Homo sapiens

<400> 6  
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 1 5 10 15

Pro Glu Ala Ser Glu Ser Thr Thr Asp Glu Asn Glu Asp Asp Ile Gln  
 20 25 30

Phe Val Ser Glu Gly Pro Ser Arg Pro Val Leu Glu Tyr Ile Asp Leu  
 35 40 45

Val Cys Gly Asp Asp Glu Asn Pro Ser Ala Tyr Tyr Ser Asp Ile Leu  
 50 55 60

Phe Pro Lys Met Pro Lys Arg Gln Gly Asp Phe Leu His Phe Leu Asn  
 65 70 75 80

Met Lys Lys Val Lys Thr Asp Thr Glu Asn Asn Glu Val Ser Lys Asn  
 85 90 95

His Cys Arg Leu Ser Lys Ala Lys Glu Pro His Phe Glu Tyr Ile Glu  
 100 105 110

Gln Pro Ile Ile Glu Glu Lys Pro Ser Leu Ser Ser Lys Lys Glu Ile  
 115 120 125

Asp Asn Leu Val Leu Pro Asp Cys Trp Asn Glu Lys Gln Ala Phe Met  
 130 135 140

Phe Thr Glu Gln Tyr Lys Trp Leu Glu Ile Lys Glu Gly Lys Leu Gly  
 145 150 155 160

Cys Lys Asp Cys Ser Ala Val Arg His Leu Gly Ser Lys Ala Glu Lys  
 165 170 175

His Val His Val Ser Lys Glu Trp Ile Ala Tyr Leu Val Thr Pro Asn  
 180 185 190

Gly Ser Asn Lys Thr Thr Arg Gln Ala Ser Leu Arg Lys Lys Ile Arg  
 195 200 205

Glu His Asp Val Ser Lys Ala His Gly Lys Ile Gln Asp Leu Leu Lys  
 210 215 220

Glu Ser Thr Asn Asp Ser Ile Cys Asn Leu Val His Lys Gln Asn Asn

225                      230                      235                      240  
 Lys Asn Ile Asp Ala Thr Val Lys Val Phe Asn Thr Val Tyr Ser Leu  
                                  245                                   250                                   255  
 Val Lys His Asn Arg Pro Leu Ser Asp Ile Glu Gly Ala Arg Glu Leu  
                                  260                                   265                                   270  
 Gln Glu Lys Asn Gly Glu Val Asn Cys Leu Asn Thr Arg Tyr Ser Ala  
                                  275                                   280                                   285  
 Thr Arg Ile Ala Glu His Ile Ala Lys Glu Met Lys Met Lys Ile Phe  
                                  290                                   295                                   300  
 Lys Asn Ile Ile Glu Glu Asn Ala Lys Ile Cys Ile Ile Ile Asp Glu  
 305                                   310                                   315                                   320  
 Ala Ser Thr Val Ser Lys Lys Thr Thr Leu Val Ile Tyr Leu Gln Cys  
                                  325                                   330                                   335  
 Thr Ile Gln Ser Ala Pro Ala Pro Val Met Leu Phe Val Ala Leu Lys  
                                  340                                   345                                   350  
 Glu Leu Val Ser Thr Ile Ala Glu Cys Ile Val Asn Thr Leu Leu Thr  
                                  355                                   360                                   365  
 Thr Leu Asn Asp Cys Gly Phe Thr Asn Glu Tyr Leu Lys Ala Asn Leu  
                                  370                                   375                                   380  
 Ile Ala Phe Cys Ser Asp Gly Ala Asn Thr Xaa Leu Gly Arg Lys Ser  
 385                                   390                                   395                                   400  
 Gly Val Ala Thr Lys Leu Leu Glu Asn Phe Pro Glu Ile Ile Ile Trp  
                                  405                                   410                                   415  
 Asn Cys Leu Asn His Arg Leu Gln Leu Ser Leu Asp Asp Ser Ile Ser  
                                  420                                   425                                   430  
 Glu Ile Lys Gln Ile Asn His Leu Xaa Tyr  
                                  435                                   440